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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 90/09446
C12N 15/55, 9/18, C12P 7/62 C11D 3/386 // C12N 15/62	A1	(43) International Publication Date: 23 August 1990 (23.08.90)

(21) International Application Number: PCT/EP90/00289

(22) International Filing Date: 19 February 1990 (19.02.90)

(30) Priority data:
89400462.1 17 February 1989 (17.02.89) EP
(34) Countries for which the regional or international application was filed: AT et al.

(71) Applicant *(for all designated States except US):* PLANT GENETICS SYSTEMS, N.V. [BE/BE]; Kolonel Bourgstraat 106, B-1040 Brussels (BE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only) :* DE GEUS, Pieter [NL/NL]; Blaauwweg 305, NL-3328 XH Dordrecht (NL).

(74) Agents: GUTMANN, Ernest et al.; Ernest Gutmann-Yves Plasseraud S.A., 67, boulevard Haussmann, F-75008 Paris (FR).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CUTINASE

(57) Abstract

A pure cutinase is secreted from *E. coli* transformed with a DNA sequence from *Fusarium solani pisi*. The pure cutinase can efficiently catalyse the hydrolysis and the synthesis of esters in aqueous and non-aqueous media, both in the absence and the presence of an interface between the cutinase and the substrates.

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CUTINASE

This invention relates to a cutinase (the "pure cutinase") free of all other hydrolytic enzymes such as lipases, which naturally occur in cells of microorganisms (e.g., fungi) that produce cutinases in nature. This invention particularly relates to a cutinase derivative (the "cutinase derivative") comprising a cutinase which: 1) has, at its N-terminal end, a foreign amino acid sequence and/or 2) is not glycosylated.

This invention also relates to the cloning and expression of a cutinase-encoding gene, preferably a derivative of the gene, in prokaryotic microorganisms, preferably in E. coli, to produce the pure cutinase, preferably the cutinase derivative, as as recombinant cutinase.

This invention further relates to novel uses of such a pure cutinase or cutinase derivative as a remarkably thermostable and active biocatalyst in processes for the hydrolysis or synthesis of esters, particularly esters of short and long chain fatty acids and/or alcohols, and of triglycerides (natural fats) in water, in water-free organic solvents or in water-free milieu where one or more reactant(s) act as the solvent.

Background of the Invention

Cutinases are hydrolytic enzymes which have heretofore been extracted from several phytopathogenic fungi and from pollen. Phytopathogenic fungi are believed to use extracellular cutinases to degrade the structural component of plant cuticle (i.e., cutin) in order to facilitate their penetration into plants (Kolattukudy, 1984). The natural mode of action of cutinases involves the hydrolysis of the ester bonds in cutin which consist of a network of esterified long-chain fatty acids and

fatty alcohols. Fungal cutinases also catalyze hydrolysis of paranitrophenylesters of varying chain lengths, as well as other esters (Kolattukudy, 1984). No other enzyme is yet known which degrades cutin, but the precise mode of action of cutinases on cutin is not known.

Cutin monomers, unique to cutin, and a soluble protein factor from fungal extracts selectively trigger transcription of cutinase-encoding genes in fungal pathogens (Podila et al, 1988). So far, studies on cutinases have focused an its role in fungal pathogenesis in plants.

Fungal cutinases have been well characterized biochemically. Most cutinases have a molecular weight ranging from 22 to 26 Kd and have similar amino acid compositions, including the presence of two disulphide bridges with no free SH-groups, one tryptophane and one or two histine residues (Lin and Kolattukudy, 1980). All fungal cutinases are glycoproteins containing a few percent O-glycosidally attached carbohydrates.

The fungal cutinases from Fusarium solani species possesses a unique NH_2-terminus, consisting of a covalently attached glucuronic acid molecule which forms an amide linkage with the NH_2-terminal glycine residue located at position 32 in the cutinase-coding sequence (Soliday et al, 1984). In vitro translation experiments show that this cutinase is synthesized as a precursor bearing a signal peptide (Flurkey and Kolattukudy, 1981). The signal sequence is 31 residues long, with a molecular weight of 3317 (Soliday et al, 1984). Comparison of the primary structure of this cutinase with that of other hydrolytic enzymes containing the classic active serine catalytic triad revealed no significant homology, and immunological cross-reactivity is observed only with cutinases from closely related fungi (Kollatukudy, 1984).

cDNA encoding a cutinase has been prepared from mRNA isolated from induced cultures of Fusarium solani pisi, and the nucleotide sequence has subsequently been determined. The nucleotide sequence of the cutinase gene showed an open reading frame interrupted by a single 51 bp intron.

Until now, cutinases have been suggested for use only for very limited purposes, despite their known ability to catalyze the hydrolysis of esters. Cutinases have been suggested for use with chemical agents such as fungicides and pesticides to obtain better penetration and adhesion of the chemical agents to softened plant surfaces (European patent publications 272002 and 197622). Cutinases also have been suggested for use with surfactants in detergents (PCT publication WO88/09367).

Chemical processes catalyzed by enzymes can offer many advantages over traditional chemical processes. Enzymes can show a remarkable selectivity, high activity and versatility and are able to catalyze a variety of transformations under mild reaction conditions in a selective way. Furthermore, enzyme-based processes are often simple (i.e., do not require cofactors), easy to control, energy efficient and hence inexpensive.

However, a number of factors have so far limited the use of enzyme-catalyzed process. The use of enzymes has generally been confined to aqueous environments, and their use in non-aqueous environments has required their stabilization and protection against the denaturing action of most organic solvents. Additionally, enzymes generally have an intrinsically high selectivity which has limited the scope of their potential applications. In this regard, it has been considered economically unattractive to process a homologous series of related compounds, using a different enzyme for each compound.

Lipases are a class of highly specialized esterases which have become widely used in enzyme-catalyzed processes. Lipases differ from other esterases in two crucial respects. They can effectively catalyze the hydrolysis of esters of long chain fatty acids and/or alcohols, which by virtue of their lipophilic character are not monomerically dispersed in water but rather are present in the form of aggregates. This implies that the enzyme is acting at an interface between the ester-substrate aggregates and the water. Secondly, the activity of lipases is markedly enhanced by the presence of such interfaces, as compared to their activity on monomeric substrates, and such lipase activity provides a special kind of reaction kinetics summarized by the term "lipolysis". A limited number of other enzymes (e.g., phospholipases A and C) also possess this special kind of kinetics, and they are commonly referred to as the "lipolytic enzymes" (Verger and De Haas, 1976).

Although lipases can be used for the hydrolysis and synthesis of esters, are active in organic solvents and have a wide substrate specificity, they still have a number of shortcomings. First, lipases are relatively large and in most cases rather unstable enzymes. Secondly, lipase-catalyzed reactions are not easy to control because lipases are highly specialized enzymes which act more efficiently on substrates in the form of aggregates, i.e., at lipid/water interfaces, rather than free in solution (Verger and de Haas, 1976). This has meant that the reaction velocity of a lipase has been a highly complex function of the precise physico-chemical parameters of the reaction. This has also meant that immobilization of lipases will strongly influence their performance because their activity on aggregated substrates is inevitably

going to suffer from diffusional limitations and will inevitably be low on monomeric free substrates (European patent publication 140542).

Summary of the Invention

In accordance with this invention is provided a pure cutinase which: 1) is free of all other hydrolytic enzymes, particularly lipases, that naturally occur in cells of microorganisms, such as fungi, producing cutinases in nature; and 2) is preferably a cutinase derivative that i) has, at its N- or amino-terminal end, a foreign amino acid sequence, preferably of at least 5 amino acids in length, and/or ii) is not glycosylated.

Also in accordance with this invention, the pure cutinase, preferably the cutinase derivative, is used as a biocatalyst, preferably for the hydrolysis or synthesis of an ester, especially a monoester or triglyceride, the esterification of a sugar, or a transesterification reaction. Such a reaction can be carried out in aqueous or non-aqueous environments, independent of the presence of aggregated forms of the reactants or lipid/water interfaces, and at relatively high temperatures.

Further in accordance with this invention is provided a recombinant gene (the "recombinant cutinase gene") coding for the pure cutinase, particularly the cutinase derivative, and comprising, in the same transcriptional unit, the following operably linked DNA fragments: 5'-P-C-T-3', preferably 5'-P-S-C-T-3', especially 5'-P-S-E-C-T-3', in which: P is a promoter that can direct the expression of the recombinant cutinase gene in a selected host, preferably a prokaryote such as E. coli; S is a signal sequence which: 1) is under the control of the promoter (P) and 2) encodes a signal peptide capable of being expressed in the host and of translocating cutinase,

expressed in the host as a fusion with the signal peptide, whereby cutinase is secreted from the host; C is a DNA sequence which: 1) is also under the control of the promoter (P), 2) codes for a mature cutinase, 3) is capable of being expressed in the host and 4) is in the same reading frame as the signal sequence (if present); E is an extension sequence which: 1) encodes an amino acid sequence or bridging sequence capable of being expressed in the host as a fusion with both the C- or carboxy-terminal end of the signal peptide and the N-terminal end of the mature cutinase, 2) is foreign to the mature cutinase, 3) is in the same reading frame as the cutinase-encoding sequence (C) and the signal sequence (S), if present, and 4) preferably has a cleavage site, within it, recognized by one or more peptidases (e.g., signal peptidases) of the host that also recognize a cleavage site in the signal peptide; and T is a 3' transcription regulation sequence which can direct the expression of the recombinant cutinase gene in the host.

Still further in accordance with this invention, the pure cutinase, preferably the cutinase derivative, is produced by culturing cells of a host that is transformed with the recombinant cutinase gene and that preferably contains a signal peptidase that recognizes the cleavage site encoded by the signal sequence (S) and the extension sequence (E).

Yet further in accordance with this invention are provided: 1) the pure cutinase, preferably the cutinase derivative, immobilized on a suitable support and 2) the use thereof for catalyzing a hydrolysis or esterification process.

Detailed Description of the Invention

This invention relates to a cutinase, preferably of natural original, especially a cutinase of fungal origin, particularly a cutinase of a Fusarium solani, quite particularly a cutinase of Fusarium solani pisi. The cutinase of this invention is particularly characterized by: 1) having the ability to hydrolyze the ester-bonds of cutin under natural conditions; 2) being a serine esterase with an active site serine present in the sequence Gly-X-Ser-X-Gly, preferably Gly-Gly-X-Ser-X-Gly; 3) having an enzymatic activity on esters which is not influenced by the presence of aggregated forms of the substrate; and preferably 4) having a molecular weight ranging from approximately 18 Kd to 30 Kd. By "aggregated forms of the substrate" is meant that the substrate is in a different phase from at least one other component of a reaction mixture containing the enzyme (i.e., that an interface is present). Examples of cutinases of this invention are those described by Ettinger et al (1987) and those from the organisms listed in PCT patent publication WO88/09367.

A cDNA of a cutinase of natural origin can be synthesized from mRNA encoding the cutinase in its natural host and then cloned in a conventional manner. See Soliday et al (1984). The cDNA can be used for the construction of the recombinant cutinase gene of this invention, which can then be incorporated in a vector capable of providing replication and expression of the recombinant cutinase gene in a suitable host microorganism. Preferred vectors are the pMa/c5-8 mutagenesis vectors described by Stanssens et al (1987) and (1989), but other vectors, such as those described by Winacker (1987), can also be used.

The selection of the host microorganism, to be transformed with the recombinant cutinase gene of this

invention, is not believed to be critical, and any host which can express the foreign cutinase-encoding sequence (C) of the recombinant cutinase gene and preferably can secrete the pure cutinase, preferably the cutinase derivative, can be utilized. A prokaryote, such as E. coli, is a preferred host for obtaining the pure cutinase in large quantities and high yields. The use of a prokaryotic host also provides the cutinase derivative of this invention in a non-glycosylated form. A preferred E. coli strain is E. coli K-12 (Bernard et al, 1979), preferably with a $lacI^q$ repressor gene (Müller-Hill et al, 1968; Zabeau and Stanley, 1982). An example of such a strain is E. coli WK6 (Zell and Fritz, 1987). However, other hosts can also be used, such as other gram-negative bacteria, gram-positive bacteria (e.g., Bacillus subtilis), yeast and filamentous fungi, which are conventionally used in industrial fermentation processes. Nevertheless, the vectors and the DNA fragments (P-S-E-T) of the recombinant cutinase gene must be compatible with the host, so as to provide expression of the recombinant cutinase gene by the host.

The transformed host microorganism can be cultured in a conventional manner, and the pure cutinase, preferably the cutinase derivative, so-produced can be recovered from the periplasmic space (e.g., in E. coli) or from the culture medium (e.g., with B. subtilis or yeast) by conventional techniques. The cutinase can then be simply purified at low cost while retaining its specific activity.

The selection of the promoter (P) in the recombinant cutinase gene of this invention is not critical and will generally vary with the host, to be transformed. Preferred promoters (P) for the expression of the recombinant cutinase gene in a host are the strong, preferably

regulatable, promoters for the host such as the following promoters for E. coli: the P_{tac} promoter (De Boer et al, 1983) the sequence of which is shown in Fig. 7; the P_{lac} promoter (Fuller, 1982); the P_{trp} promoter (Martial et al, 1979); the lambda P_L promoter (Bernard et al, 1979); and the P_R promoter (Zabeau and Stanley, 1982).

The selection of the signal sequence (S) in the recombinant cutinase gene of this invention also is not critical and generally will vary with the host, to be transformed. The signal sequence (S) provides a translation initiation site for the recombinant cutinase gene and encodes a signal peptide having the necessary functional sequence for exporting the recombinant cutinase, preferably with all or part of its N-terminal foreign bridging sequence, from the host cell. Preferably, the recombinant cutinase is transported by the signal peptide into the periplasmic space of the host, with clipping of the signal peptide from the recombinant cutinase by one or more peptidases (e.g., signal peptidases) of the host. In this regard, the signal peptide, encoded by the signal sequence (S), is an amino acid sequence which is normally fused, in a host organism, to the N-terminal end of a protein or subunit of a protein, produced in the host. The signal peptide is normally responsible for the translocation of the protein or subunit to the periplasmic space of a host such as E. coli or into the medium containing a host such as B. subtilis, following the production of the fused signal peptide and protein or subunit in the host. The signal peptide is normally separated proteolytically from the protein or subunit at a cleavage site at the C-terminal end of the signal peptide during translocation. An example of a suitable signal sequence (S) for use in the recombinant cutinase gene of this invention in E. coli is

the signal sequence coding for the phoA signal peptide (Michaelis et al, 1983) which is shown in Fig. 7, but other signal sequences, such as those described by Watson (1984), can also be used in E. coli. The signal peptide, encoded by the signal sequence (S), can be endogenous or heterologous to the encoded cutinase and/or endogenous or heterologous to the host.

Although the use of a signal sequence (S) is not absolutely necessary, its use is preferred in the recombinant cutinase gene of this invention in order to facilitate the purification of the cutinase product, during which other hydrolytic enzymes may have to be removed. However, if desired, a host can be transformed with just a cutinase-encoding DNA sequence (C) under the control of a suitable promoter (P), and cutinase, expressed by the transformed cell intracellularly, can be obtained by lysing the host cell and then separating the pure cutinase from the other hydrolytic enzymes in the host cell, as well as the other contents of the host cell.

The extension sequence (E), although also not necessary, is preferred in the recombinant gene of this invention. The extension sequence (E) can also vary, depending on the host, to be transformed. The extension sequence encodes a bridging sequence linking the C-terminal end of the signal peptide, encoded by the signal sequence (S), and the N-terminal end of the mature cutinase, encoded by the cutinase-encoding sequence (C). The length of the bridging sequence is not believed to be critical and can suitably be from 1 to 100 amino acids in length. The presence of the extension sequence (E) has been found to provide optimal expression of the cutinase-encoding sequence (C) in a host microorganism, such as E. coli, as measured by the export or secretion of a cutinase derivative of this invention.

Preferably, the extension sequence (E) also encodes, in the bridging sequence, a peptidase recognition sequence which is recognized by one or more peptidases (e.g., signal peptidases) in the host cell. This peptidase recognition sequence contains a cleavage site that can be efficiently cleaved by such peptidases during the secretion of the recombinant cutinase from the host cell. It is believed that an efficient cleavage of the bridging sequence from the recombinant cutinase will generally improve the secretion process and thus the yield of the desired pure cutinase, preferably the cutinase derivative, of this invention. Such a peptidase recognition sequence can be entirely encoded by the extension sequence (E), or it can be partially encoded by the 3' parts of the signal sequence (S) and partially encoded by the 5' parts of the extension sequence (E), or it can be partially encoded by the 3' parts of the extension sequence (E) and the 5' parts of the cutinase-encoding sequence (C). The actual cleavage site in the peptidase recognition sequence can thus be located within the bridging sequence, the signal peptide or the mature cutinase or on the borders between any two adjacent elements. It goes without saying that, if no extension sequence (E) is used, any such peptidase recognition sequence should be encoded by the 3' parts of the signal sequence (S) and/or the 5' parts of the cutinase-encoding sequence (C). In this regard, the parts of the signal sequence (S) and/or the cutinase-encoding sequence (C), that encode the peptidase recognition sequence, can be changed (e.g., by means of site-directed mutagenesis) so as to encode a more efficient peptidase recognition sequence.

The peptidase recognition sequence of the bridging sequence, encoded by the extension sequence (E), preferably is recognized by the same, one or more

peptidases of the host which are involved, in vivo, in cleaving the signal peptide from the recombinant cutinase. However, if desired, the peptidase recognition sequence of the bridging sequence can be recognized by one or more peptidases, not found within the host but used, in vitro, to cleave the bridging sequence from the recombinaIOnt cutinase after secretion of the cutinase, fused to the bridging sequence, from the host. The exact location of the cleavage site of the peptidase recognition sequence on the bridging sequence is not critical and can be located at the C-terminal end of the bridging sequence (i.e., at the N-terminal end of the cutinase). However, if any portion of the bridging sequence (including a C-terminal portion of the signal peptide which has not been cleaved off the bridging sequence) is to be left on the recombinant cutinase, after in vivo or in vitro cleavage of the peptidase recognition sequence of the bridging sequence, the length of the remaining portion of the bridging sequence on the N-terminal end of the mature cutinase is not believed to be critical. In this regard, that remaining portion of the bridging sequence can be 1-50 amino acids in length, preferably about 10-35 amino acids in length, especially about 12-25 amino acids in length.

Any part of the bridging sequence which remains attached to the mature cutinase (i.e., is between the cleavage site of the peptidase recognition sequence of the bridging sequence and the cutinase), after the bridging sequence has been cut within its peptidase recognition sequence, preferably does not affect significantly the enzymatic properties of the resulting recombinant cutinase. The amino acid sequences of two examples of bridging sequences of this invention, encoded by extension sequences (E), which can be used in the recombinant

cutinase gene of this invention, are shown in Figure 2. Preferred extension sequences (E) encode bridging sequences which do not naturally occur on (i.e., are foreign to) the naturally occurring, mature cutinase encoded by the DNA sequence (C).

The selection of the 3' transcription regulation sequence (T) also is not critical and will generally vary with the host, to be transformed. This sequence serves in prokaryotes to terminate transcription and also serves in plasmid for stability purposes. The phage fd transcription terminator, preferably two copies in tandem (Botterman and Zabeau, 1987), is preferred for this purpose, but of course, other transcription terminators, well known to those skilled in the art, can also be used.

A preferred cutinase-encoding sequence (C) in the recombinant cutinase gene of this invention encodes a naturally-occurring cutinase, preferably a fungal cutinase, especially an F. solarium cutinase. A particularly preferred cutinase-encoding sequence (C) is the DNA sequence starting at the codon encoding position Gly_{32} in the natural cutinase-encoding sequence from Fusarium solani pisi (Soliday et al, 1984) (see Figure 1). However, any DNA sequence coding for a cutinase, including a cutinase of pollen origin, can be used in this invention.

It has been found that a pure cutinase of this invention, particularly a cutinase derivative of this invention, possesses surprising properties which make it superior to lipases for use in enzyme-catalyzed reactions. In this regard, the thermostability of cutinases in aqueous solution is higher than that of any lipase reported so far, including those from Candida described in PCT patent publication WO 88/02775. Furthermore, although a cutinase's natural substrate is cutin, which is an

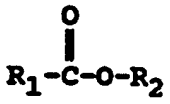
insoluble polymeric polyester, the activity of the enzyme was found to be independent of the presence of substrate aggregates or lipid/water interfaces in reaction mixtures. This is in contrast to the activity of lipases. Indeed, unlike lipases, the activity of cutinases remains high and independent of the presence of interfaces. As a result, the use of a cutinase in industrial reactions can be more easily and simply controlled than the use of lipases. Also, because of cutinase's simple reaction kinetics, reaction rate control and immobilization are greatly facilitated since there is no need to stably maintain, for example, emulsions with well defined physico-chemical parameters. In addition, an immobilized cutinase can be used with substrates which normally form aggregates but which can be presented to the cutinase as monomers (by changing the solvent). This will result in rapid diffusion of the substrate to the enzyme and rapid catalytic conversion by the enzyme. By comparison, immobilization of a lipase presents complicated process problems and most often leads to a reduction in enzymatic activity (European patent publication 010542; and Goderis et al, 1987).

The use of the pure cutinase for the catalysis of a variety of reactions is of further advantage because such an enzyme is highly stable and active both in aqueous and non-aqueous environments, and under appropriate conditions, it offers remarkable efficiency and selectivity. One of the most neglected aspects of enzyme technology is enzyme stability in organic solvents. However, the pure cutinase of this invention is exceedingly stable under conventional hydrolysis conditions, allowing repeated use of the enzyme and thus making the economics of such a process attractive.

The pure cutinase can be used in accordance with this invention for the hydrolysis of a variety of ester

substrates including monoesters, such as p-nitrophenylbutyrate, and triglycerides, such as trioleine and tributyrine. Unlike an aspecific esterase, the cutinase can also catalyze the rapid hydrolysis of long-chain triglycerides, such as trioleine, in emulsion form. The substrate dependence profile of the catalytic activity of the cutinase on tributyrine shows that its enzymatic activity is not influenced by the formation of an interface. At the substrate concentration where aggregates of tributyrine are formed, no appreciable increase in enzyme activity can be noticed, quite in contrast to what is known for lipases (Macrae, 1983; Verger and de Haas, 1976).

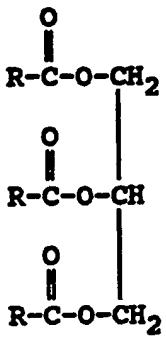
The hydrolysis of monoesters is an important intermediate step in a large number of industrial processes, for example in the manufacture of flavorings. Monoesters have the following general formula:



in which R_1 and R_2 can be aliphatic and non-aliphatic groups, preferably of at least 15 carbon atoms, which can be saturated or unsaturated, straight chain or branched chain, cyclic or heterocyclic.

In accordance with this invention, a monoester, such as ethylcaprylate, can be rapidly hydrolyzed in the presence of the pure cutinase into ethanol and caprylic acid.

The mild hydrolysis of natural fats (triglycerides) and oils with the pure cutinase to produce free fatty acids and mono- and diglycerides also represents an important step in commercial processes. Triglycerides are triesters of the alcohol, glycerol, and fatty acids having the following general formula:



in which R represents an alkyl chain.

Animal and vegetable fats are esters of long-chain fatty acids and glycerol (glyceryl esters). Glyceryl esters, which are liquid at room temperature, are commonly called oils. The pure cutinase, particularly the cutinase derivative, of this invention is a highly thermostable enzyme which can be used effectively at relatively high reaction temperatures. This has made the enzyme well-suited for modification of some fats which melt at high temperatures.

Cutinase-catalyzed production of mono- and diglycerides in accordance with this invention holds great promise as an alternative to traditional chemical methods of synthesizing compounds because of the mild reaction conditions and low by-product formation in cutinase-catalyzed reactions. An additional advantage of such a process resides in the fact that highly unsaturated triglycerides can be used as a starting material, directly, without prior hydrogenation. The resulting monoglycerides can be used in food and pharmaceutical industries for emulsification, aeration, starch complexing or crystal modification (Zaks et al, 1988). Because such products are obtained by enzymatic catalysis, rather than conventional fat splitting, they have better odor, and they are cheaper to make.

The cutinase-catalyzed hydrolysis of triglycerides, especially those of natural origin, at high pH in accordance with this invention can be used to produce cleaning agents, such as laundry detergents and other specialized fat dissolving preparations, such as cosmetic compositions and shampoos. The pure cutinase is particularly well suited for such processes because of its general stability, particularly thermostability.

The pure cutinase can also be used for the direct synthesis of esters starting from alcohols and organic acids (e.g., fatty acids). This derives from the reversibility of the cutinase reaction.

The pure cutinase can further be used to catalyze synthetic transformations, such as transesterification reactions, including alcoholysis, acidolysis and interesterification reactions. Transesterification of fats and oils can be used to produce new mixtures of fats, fatty acids, and esters with desirable properties. The ability to produce novel fat mixtures, using a cutinase, is of interest to the oil and fat industry where the transesterification process is used to modify the composition, and hence the physical properties, of mixtures of fats and esters. The use of more specific enzyme catalyst for transesterification processes also provides additional flexibility in controlling the structure of the resulting modified fats, a flexibility which is not available with conventional chemical transesterification processes.

Also in accordance with this invention, the pure cutinase is used to catalyze alcoholysis in a water-free milieu. In such a process, one alcohol displaces another alcohol from an ester.

Further in accordance with this invention, useful reactions, such as transesterification, which normally

would not occur to an appreciable extent in water, can be catalyzed efficiently with the pure cutinase in organic solvents. Also, in hydrolytic reactions where H_2O is one of the reactants, the equilibrium can be shifted in non-aqueous media to favor condensation products (e.g., esters).

An important use of the pure cutinase is in the esterification of sugar compounds. The cutinase is able to catalyze the esterification of sugars in polar or hydrophylic organic solvents such as pyridine and dimethylformamide (DMF) in which sugars are reasonably soluble. Therefore, the cutinase can be used for the synthesis of sugar compounds, either directly or by a transesterification-type of reaction in such solvents. Moreover, carbohydrates represent a target for regioselective modification of their multiple hydroxyl groups via an enzymatic transesterification reaction. This is important for the production of biological surfactants. Biological surfactants possess a number of potential advantages over their chemically manufactured counterparts, including lower toxicity and biodegradability.

The pure cutinase can further be used in the stereospecific resolution of a racemic mixture. In this regard, the cutinase can be used for the regioselective esterification of primary and secondary alcohols, as well as for the stereospecific conversion of esters into fatty acids and alcohols and fatty acids and alcohols into esters and for the production of pure, optically active compounds. The advantages in chemo-, regio- and stereospecificity, exhibited by the pure cutinase, can be exploited in the synthesis and resolution of a wide variety of optically active acids, alcohols and esters. The stereoselectivity of the cutinase also can be

exploited in preparing beta-blockers in optically active form (Kloosterman et al, 1988). Another example of a stereospecific action of the cutinase is its use for catalyzing the hydrolysis of (R,S)-2-chloropropionic acid esters to produce useful intermediates for the production of herbicides.

The reversibility of a cutinase-catalyzed reaction enables the enzyme to be also used in the direct formation of esters from alcohols and fatty acids. The pure cutinase can thus be used for the direct synthesis of esters in an organic (water-free) solvent (e.g., heptane) starting from an alcohol (e.g., butanol) and a fatty acid (e.g., lauric acid).

The pure cutinase also holds great promise as catalyst for the production of chiral organic compounds such as glycidol esters or optically active cyclohexanepolyol derivatives (Dumortier et al, 1989). Esters of glycidol are used for the production of β-blockers in the pharmaceutical industry (Kloosterman et al, 1988).

Of course, for any use of the pure cutinase, preferably the cutinase derivative, of this invention, conventional excipients, adjuvants, stabilizers and other enzymes can be added to it to enhance its stability or its usefulness for a particular purpose. For example, enzymes such as proteases, amylases, and/or cellulases can be added to the pure cutinase for providing a detergent composition.

In order to catalyze different types of reactions, the pure cutinase, preferably the cutinase derivative, can be used as such in batch or can be immobilized in a conventional manner, for example as described in European patent application 86/401933.6.

The traditional use of industrial enzymes has been in batch processes, employing soluble enzymes and aqueous

substrates. Recovery and reuse of the enzyme catalyst has been extremely difficult under these circumstances, and the enzyme has therefore normally not been recovered from batch reaction mixture.

Many of the difficulties encountered in using soluble enzymes can be overcome by the use of immobilized enzyme preparations which are generally more stable, reusable and available for continuous processing. Immobilization of the pure cutinase on a solid support allows one to operate in non-aqueous media. As a result, one can carry out, besides hydrolysis reactions in aqueous media, synthetic transformations such as alcoholysis and esterification in non-aqueous media. Furthermore immobilization of the enzyme allows one to use the catalyst in a continuous column operation, with substrates in the liquid and/or gaseous phase. In the case of reactions with substrates in a gas phase, the enzyme can also be directly used in the solid phase, e.g., as a lyophilized powder packed in a reactor.

The pure cutinase can easily be immobilized on polyvinylpyridine-coated glass substrate according to the procedure disclosed in European patent application 86/401933.6. Another way to immobilize the cutinase involves the specific modification of the cutinase so as to allow covalent coupling of the enzyme to a suitable carrier. Of course, other immobilization procedures can be used, for example as described in Methods of Enzymatic Analysis, Ed. H. Bergmeyer, Third Ed., Vol. 1, Verlag Chemie, 1983, whereby the immobilized cutinase preparation preserves its stability and enzymatic activity.

It has been found that the pure cutinase, preferably a cutinase derivative, of this invention can be effectively used as a substitute for a lipase in many lipase-catalyzed reactions such as those described, for example, in

European patent publication 232933 and UK patent publication 577933. The pure cutinase can act as an effective catalyst on a wide variety of non-natural substrates composed of esters, alcohols and fatty-acids or derivatives thereof, under a wide variety of reaction conditions, both in aqueous and non-aqueous environments.

The following Examples illustrate the invention. The figures, referred to in the Examples, are as follows:

Fig. 1 Nucleotide sequence of the cDNA of the cutinase gene of Fusarium solani pisi. The derived amino acid sequence of the natural cutinase from F. solani pisi is also shown. The putative NH_2-terminal glycine residue at position 32 is indicated by an arrowhead. The active site serine-containing sequence Gly-X-Ser-X-Gly, which is typical for all cutinases, is underlined.

Fig. 2 Amino acid sequences of different bridging sequences for fusing a signal peptide to a cutinase.

Fig. 3 Thermoinactivation of cutinase in aqueous buffer as a function of temperature.

Fig. 4 Kinetics of thermoinactivation at 80°C and pH 7.5, 6.0, 8.5.

Fig. 5 The dependence of trioleine hydrolysis as a function of pH.

Fig. 6 The dependence of cutinase activity on the presence of substrate aggregates.

Fig. 7 Sequence of the tac-promoter followed by the sequence coding for the phoA signal peptide, the amino acid sequence of which is also shown.

Cloning Procedures

Unless otherwise stated in the Examples, all procedures for making and manipulating recombinant DNA were carried out by the standardized procedures described in Maniatis et al, "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory (1982).

All site-directed mutagenesis was performed by oligonucleotide-directed construction of mutations by the gapped duplex DNA method using the pMa/c vectors described by Stanssens et al (1987, 1989). Appropriate oligonucleotides were designed according to the general rules outlined by Kramer and Fritz (1988) and synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981) on an Applied Biosystems 380A DNA synthesizer.

Reaction products in non-aqueous environment

To catalyze reactions in essentially water-free medium, the cutinase enzyme, preferably 10 mg, was lyophilized from a suitable buffer of pH preferably above 7 but below 11 and of low ionic strength. Alternatively, the enzyme could have been precipitated from an aqueous buffer by the addition of 2 volumes of ice-cold acetone, followed by centrifugation for 10 minutes at 5000 g and drying under a stream of nitrogen. The dry enzyme was then dispersed in an organic solvent containing a substrate and shaken vigorously at appropriate temperatures for a defined period of time, after which the reaction mixture was extracted with chloroform: methanol (2:1). The reaction products were examined by thin layer silica gel chromatography ("TLC") (Kates, 1986). Spots were visualized with iodine vapor by baking and/or by detection with potassium periodocuprate spray.

Enzyme activity units

The enzyme activity was routinely determined in three assays, using: trioleine and tributyrine emulsions in an automated pH-stat consisting of a Methrom 614 Impulsomat, a Methrom 632 pH meter, a Methrom 665 Dosimat (with 20 ml unit) and a Methrom E580 Labograph (Methrom Ltd., Haeresau, Switzerland); or p-nitrophenylbutyrate in monomeric solution as a chromogenic substrate in a Kontron-Uvikon 810P spectrophotometer (Kontron A.G., Switzerland).

In the case of the pH-stat, a unit is defined as the amount of enzyme which liberates 1 umol of titratable fatty acid per minute under a given set of reaction conditions. Corrections are made for the pKa of a fatty acid in water, which under certain conditions is an apparent pK_a and may be as high as 7 for oleic acid (Benzonana and Desnuelle, 1969). Stable emulsions with trioleine always require addition of a stabilizer such as arabic gum. Tributyrine can be used as such, under vigorous stirring.

In the case of p-nitrophenylbutyrate, 1 unit is defined as the amount of enzyme causing an increase in absorbance of 1 in one ml PBS (Phosphate Buffer Saline) per minute at 25°C, at a substrate concentration of 0.5mM and at a wavelength of 405nm.

The assay on trioleine measures true lipase activity, while that on p-nitrophenylbutyrate measures non-specific esterase activity. Tributyrin is intermediate between these two categories.

Care was taken when comparing activities under different conditions for enzymes active on interfaces, since it is known for lipases that small changes in interfacial properties cause large changes in activity. In the case of cutinase, reaction conditions were always

carefully controlled and standardized. Never were two activities compared from different assay conditions.

Example 1: The production and purification of a recombinant cutinase

As described below, the cDNA of the cutinase from Fusarium solani pisi (Fig. 1) was cloned and subsequently used for the synthesis of active enzyme in E. coli cells.

A vector was constructed which allowed the easy transfer and stable propagation of the cutinase sequence (Figure 1) in E. coli. Recombinant cutinase genes were ligated between the EcoRI and SalI sites of pMa5-8 (Stanssens et al, 1987). The recombinant cutinase genes consisted (in 5' to 3' direction) of: the P_{tac} promoter (P); the phoA signal sequence (S) as shown in Fig. 7 (where the EcoRI site is also shown); an extension sequences (E) coding for one of the bridging sequences shown in Fig. 2; a cutinase-encoding cDNA sequence starting from the codon for Gly-32 and ending with the SalI site as shown in Fig. 1; and the 3' transcription regulation sequence (T) from phage fd. The pMa5-8 vectors, harboring the recombinant cutinase genes, were directly used for site-directed mutagenesis.

The presence of the signal sequence (S), encoding a signal peptide with a cleavable site at or after its attachment to the bridging sequence encoded by the extension sequence (E), caused export of a recombinant cutinase derivative into the periplasmic space of the E. coli cells.

The bridging sequence encoded by the extension sequence (E) was chosen so as to allow good processing of the signal peptide and good enzymatic activity of the resulting cutinase derivative. In this regard, the bridging sequence consisted of parts of the natural amino

acid sequence preceding the Gly_{32} in the amino acid sequence encoded by the cutinase gene of F. solani pisi. From a comparison of the cutinase's natural signal peptide (amino acids 1-31) in Fig. 1 with those of other exported proteins described in Watson (1984), it appeared that the most likely signal-peptidase cleavage site in cutinase was located not before Gly_{32} but rather around the residue at position 17.

Therefore, bridging sequences were constructed from the signal peptide naturally associated with the F. solani cutinase. More specifically, the residues Leu17 to Pro22 (numbering as in Fig. 1) were selected as providing the N-terminal part of the bridging sequence. However, to improve the efficiency of cleavage and of secretion, the Leu17 was changed to an alanine residue. This was done by site-directed mutagenesis of the corresponding nucleotide sequence. This led to the development of the extension sequences coding for the bridging sequences E1 and E3 as shown in Fig. 2. The use of recombinant cutinase genes with these E sequences resulted in variable yields of secreted proteins, but large portions of the cutinase derivatives (100% in the case of E2) were still produced in inclusion bodies. Moreover, determination of the N-terminal residues of the secreted cutinase derivatives showed that cleavage took place at different sites (Table 1).

In order to further improve the cleavage and secretion, it was attempted to optimize the presumed peptidase recognition sequence by changing the C-terminal end of the signal peptide. More particularly, the three C-terminal amino acid residues Thr-Lys-Ala (see Fig. 7) were changed to Ala-Asn-Ala by means of site-directed mutagenesis of the corresponding nucleotide sequence. The new signal sequence (designated as S* - Table I), when

combined with the extension sequence E3, resulted in a complete secretion of the cutinase derivatives in high yields, but cleavage was apparently more efficiently restricted to one particular cleavage site so that a more uniform cutinase derivative could be obtained (Table I).

Yields of cutinase in E. coli were found to be heavily dependent on the sequence of the P-S-E-C-T part of the vector, more particularly of the S-E part, but in preferred examples, yields were near 1 gram per liter of culture broth (See Figure 2 and Table I).

E. coli strain WK6, harboring plasmid pMa5-8 containing the recombinant cutinase genes of this invention, was grown in 2 liter flasks in IXTB medium (Tartof and Hobbs, 1988) at 25°C-30°C in the presence of 100 ug/ml ampicillin, with vigorous shaking (150 rpm) to an OD at 600 nm of 10-12. Then, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 10uM, and the incubation continued for another 12-16 hours. When no more significant increase of the level of cutinase could be observed, the cells were harvested, and resuspended in buffer containing 20% sucrose at 0° C. The cells were spun down again and resuspended in cold water. Cell debris was removed by centrifugation, the supernatant was acidified to pH 4.8 with acetic acid (HAc) and left overnight at 4° C, and the resulting precipitate was removed. A better than 75% pure cutinase preparation, essentially free of endogenous lipases and suited for most applications of this invention, was obtained at this stage by means of ultrafiltration and freeze drying. The yield and the pronounced influence thereupon of the E-sequence (Figure 2) are given in Table I. IXTB growth medium for E. coli WK6 consisted of:

0.017 M KH_2PO_4
0.072 M K_2HPO_4
12 g/l Bacto-tryptone
24 g/l Bacto-yeast extract
0.4% glycerol (V/V)

Table I: Cutinase yields from induced E. coli WK6

Construct	Yield of secreted cutinase derivative g/l	units*/l	N-termini of secreted cutinase derivative (+ ratio)
S-E1-C	2.0	50	APTSN:ATASA 3:2
S-E2-C (= S-C)	0.01	0.25	not determined
S-E3-C	0.9	22.5	APTSN:PTSN 2:1
S*-E3-C	not determined		APTSN:PTSN 9:1

* 10^6 p-nitrophenylbutyrate units as previously defined

Thus, by selecting an optimal extension sequence (E) between the signal sequence (S) and the cutinase-encoding sequence (C) provides a very efficient and easy way to obtain large quantities of over 75% pure cutinase which can be easily formulated as a dry or liquid preparation.

If needed, the cutinase can be purified to homogeneity (i.e., better than 95% pure) by collecting the enzyme from the acidified E. coli extract onto SP-Sephadex, elution with buffer at pH 8 and passage of the concentrated basic solution through a suitable volume of DEAE-cellulose (Whatman DE-52). The DEAE flow-through can be directly applied to a Q-sepharose HP (Pharmacia) column and eluted with a salt gradient which yields a homogeneous cutinase preparation typically with better than overall 75% yield.

Example 2: Thermostability

The thermostability of the purified cutinase was determined qualitatively by preincubating the enzyme in 25mM phosphate buffer for 15 min at different temperatures at pH 7.5 at 0.1 mg/ml concentration. From the data in Figure 3, it is seen that the enzyme is stable well up to 70° C. Quantitive data were obtained by incubating the enzyme at 80° C in phosphate buffer at pH 7.5 at 0.1 mg/ml concentration for different periods of time followed by the determination of the residual activity of the enzyme. This experiment was repeated at pH 6.0 in 25 mM acetate buffer and at pH 8.5 in 25 mM borate buffer. The log of the residual activity on p-nitrophenylbutyrate was plotted against time in Figure 4.

Figure 4 shows that cutinase is most stable at its isoelectric point, pH 7.5 or slightly below, but that its stability is less at pH 8.5. At pH 7.5 the half-life of the enzyme at 80° C is calculated as 150 minutes, higher than that of an reported lipase so far. The thermoinactivation at pH 7.5 and 80°C was irreversible and followed pseudo-first order reaction kinetics (Figure 4). The half life of the enzyme under low-water conditions or as immobilized formulation is much longer than when measured in an aqueous environment. This is generally observed for many enzymes (PCT patent publication WO 88/02775) and is also true for cutinase.

Example 3: Temperature optimum of cutinase

Due to its high stability at elevated temperatures, cutinase can be used very effectively for processes in which a high temperature is desirable, for instance in processes which contain high melting fats. As an example of the use of cutinase at high temperature, the activity of the enzyme in the synthesis of an ester was measured at

increasing temperatures. At temperatures above 60° the enzyme still performed optimally.

Example 4 : Cutinase kinetics

Enzymes acting on interfaces usually show complicated kinetic behavior (Verger and de Haas, 1976). In the case of a lipase, this cause difficulties in regulating enzymatic activity during industrial processes and in maintaining good catalytic activity when the enzyme is immobilized. As an example of the simple and straightforward kinetics of cutinase, the degradation of tributyrine as a function of substrate concentration is shown in Figure 6. Measurements were made in 1mM Tris buffer, pH7.5 at 30°C. In contrast to the behavior of a lipase, the cutinase activity is not affected by the presence of interfaces as the tributyrine concentration passes the point at which an emulsion starts to form, as indicated by an arrow in Figure 6.

Example 5 : Immobilization of cutinase

Cutinase can be readily and very efficiently immobilized on, for example, polyvinylpyridine-coated glass as described in European patent application 86/401933.6.

A solution of 0.1 mg/ml cutinase in 25 mM tris buffer of pH 8.5 was contacted with the coated glass for sufficient time to allow saturation of the glass surface with the enzyme. Immobilization of the enzyme could then be enhanced by either drying the glass or by treatment with 1% or 2% glutaraldehyde solution, followed by extensive washing with glycine buffer of pH 7.5.

The so-obtained immobilized enzyme preparation was highly active and could be repeatedly used, for example, for the hydrolysis of p-nitrophenylbutyrate.

Similarly, cutinase is immobilized on controlled-pore glass beads according to European patent application 86/401933.6. In this regard, a cutinase solution is continuously circulated through a column containing glass-beads until no further reduction in the cutinase concentration of the solution is measured. Then, the glass beads, coated with the enzyme, is treated as described above.

In accordance with a preferred embodiment of the invention, the recombinant cutinase of Example 1 is specifically modified for immobilization purposes by an extra cysteine residue in the bridging sequence encoded by the extension sequence E, thus providing the recombinant cutinase with a free thiol-group (sulphydryl-group), which can be used by a simple oxidation to covalently couple the enzyme to any carrier material containing free thiol groups.

Example 6: Hydrolysis of Trioleine at different pH

As an example of cutinase's ability to catalyze the hydrolysis of long chain triglycerides (natural fats), its activity was determined on a trioleine emulsion in the pH-stat at different pH. Trioleine hydrolysis is measured preferably in 10 ml 0.5 mM buffer containing 3% trioleine emulsified in 3% arabic gum, at pH preferably above 8.0 usually at 30°C. One unit is defined as the amount of enzyme liberating 1 umol of titratable acid under the specified reaction condition per minute. For pH below 8, correction must be made for the apparent pKa of oleic acid. No other additions, like Ca^{++} ions or bovine serum albumin, were found necessary for activity. Figure 5 shows the specific activity of cutinase on trioleine at different pH. An apparent basic pH optimum was found, and the level of activity was comparable to that of many true

lipases. Thus, it can be concluded that cutinase has a very good activity on "natural" fats.

Example 7: Transesterification by cutinase

As an example of an transesterification type of reaction, the alcoholysis of trioleine with hexadecanol is carried out. The reaction is performed as follows : 10 mg of dry cutinase, obtained by acetone precipitation, was dispersed in 1 ml of trioleine containing 1 mmol of hexadecanol at 45° C. The reaction was incubated at 45° C, to prevent solidification, under vigorous stirring for more than 2 hours, preferably overnight. No special precautions were taken to remove most of the water that might be present in the reactants. After this incubation the products were examined by TLC. It was shown that about 50% of the hexadecanol was converted into hexadecyloleate ester, by comparing the spots on the TLC with those of reference compounds.

An important property of cutinase is its ability to act on secondary alcohols, which greatly enhances the scope of applications of the enzyme. As a further example, it is shown that cutinase readily catalyzes the alcoholysis of 1-monooleine by 2-propanol in heptane. 5 mg monooleine was dissolved in 1 ml heptane containing 0.2 M 2-propanol. The mixture was incubated with 5 mg cutinase for 4 hours at room temperature. TLC analysis showed almost complete conversion of the monoglyceride into about 60% isopropyl-oleate and 40% free oleic acid. Since no special attention was paid to remove most of the water present in the reactants, this proves the good activity of cutinase on secondary alcohols.

Example 8: Stereoselectivity of cutinase

To demonstrate the stereoselectivity of cutinase, a racemic mixture of (R,S)-glycidylbutyrate was selectively cleaved by cutinase, using different nucleophiles and solvents. Therefore, (R,S)-glycidylbutyrate was subjected to hydrolysis in aqueous buffer and to alcoholysis using various alcohols in various organic solvents. It was shown that under appropriate reaction conditions, cutinase can be used to resolve the stereoisomers of (R,S)-glycidylbutyrate with high selectivity.

Cutinase was also used to selectively hydrolyze one of the isomers from a racemic mixture of octyl-2-chloropropionate. Its stereoselectivity was demonstrated by measuring the optical rotation of the resulting mixture. Depending on the extent of conversion, more than 95% enantiomeric excess could be obtained.

Example 9: Ester synthesis by cutinase in organic solvent

Cutinase can be used for the direct synthesis of esters from carboxylic acids and alcohols in dry organic solvents. As an example of this useful property of the enzyme 0.2 M lauric acid and 0.2 M n-butanol in heptane were incubated with freeze-dried cutinase (10 mg/ml) for 4 hours at room temperature. Analysis by TLC revealed a more than 50% conversion of the reactants into the butyl laurate product, even though no special attempts were made to remove the water released during the reaction.

Example 10: The esterification of sugar-alcohols

10mg dry cutinase was dispersed in 1 ml of pyridine containing 0.1 mmol of either glucose, maltose or sucrose, and 0.2 mmol of trichloroethylbutyrate. After 16 hrs reaction at room temperature, pyridine was removed by evaporation and the residue extracted with chloroform:

methanol (2:1). Products were analyzed on TLC, with heptane/ether/acetic acid (60:40:2) as solvent. Sugar positive esters could be detected in all cases.

Needless to say, this invention is not limited to the transformation of a specific host microorganism or the use, for this purpose, of a recombinant cutinase gene containing any specific promoter (P), signal sequence (S), extension sequence (E), cutinase-encoding sequence (C) and/or 3' transcription regulation sequence (T) of this invention, or the use of the resulting pure cutinase, particularly a cutinase derivative, of this invention in a specific enzyme-catalyzed process. In this regard, equivalents of the specific elements and steps, used in the foregoing Examples, will be readily apparent to those skilled in the art in view the disclosure herein of the invention. For example, the DNA sequence of Fig. 1 can be easily modified by: 1) replacing some codons with others that code either for the same amino acids or for other amino acids; and/or 2) deleting or adding some codons; provided that such modifications do not subtantially alter the enzymatic properties of the encoded cutinase.

References

- Beaucage S.L. and Caruthers M.H., Tetrahedron Letters 22, 1859-1862, 1981.
- Benzonana, G. and Desnuelle, P., Biochemical and Biophycical Acta 164, 47-58, 1968.
- Bernard, H., Remaut, E., Herschfield, M., Das, H., Helsinki, jD., Yanowski, C. and Franklin, N.,Gene 5, 59-76, 1979.
- Botterman and Zabeau (1987) DNA 6:583-591
- De Boer, H., Comstock , L. and Vasser, M., Proc. Natl. Acad. Sci. USA, 80, 21-25, 1983.
- Dumortier L., Van Der Eyken J. and Vandewalle M., Tetrahedron Letters 30:3201-3204, 1989
- Ettinger, W.F., Thrukal, S.K. and Kolattukudy, P.E., Biochemistry, 26:7883-7892, 1987
- Flurkey, W. and Kolattukudy, P., Arch. Biochem. Biophys. 212, 154-162, 1981.
- Fuller, F., Gene 19, 43-54, 1982.
- Goderis, H. et al, Biotechnology and Bioengineering, XXX, 258-266, 1987.
- Kates, M. in "Techniques of Lipidology", 2nd edition, ed. R. Burton and P. van Knippenberg, Elsevier, 1986.
- Kloosterman, M., Elferink, V., Van Iersel, J., Roskam, J.-H., Meyer, E., Hulshof, L. and Sheldon, R., TIBTECH 6, 251-255, 1988.
- Kolattukudy, in "Lipases", Börgstrom and Brockman, eds, Elsevier, North Holland, pp. 471-504, 1984.
- Kramer W. and Fritz H.J., Methods in Enzymology 154, 350-367, 1968.
- Lin, T. and Kolattukudy, P., Eur. J. Biochem 106, 341-351, 1980.
- Macrae, A. in "Microbial Enzymes and Biotechnology", 225-250, ed. by Fogarty, W.R., Applied Science Publishers, 1983.

- Martial, J., Hallewel, R., Baxter, J. and Goodman, H., Science 205, 602-607, 1979.
- Michaelis et al, J.Bacteriol. 154:366, 1983
- Müller-Hill B., Crapo L. and Gilbert W., PNAS USA 59, 1259-1264, 1968.
- Podila, K., Dickman, M. and Kolattukudy, P., Science 242, 922-925, 1988.
- Soliday, C.L., Flurkey, W.H., Okita, T.W. and Kolattukudy, P.E., Proc. Natl. Acad. Sci. USA 81, 3939-3943, 1984.
- Stanssens et al. (1987) Manual EMBO Laboratory Course 'Directed Mutagenesis and Protein Engineering' held at Max-Planck Institute für Biochemie, Martinsried, West-Germany, July 4-18, 1987
- Stanssens et al. (1989) Nucleic Acids Research 17:4441-4454
- Tartof K.D. and Hobbs C.A., Gene 67, 169-182, 1988.
- Verger,R. and de Haas, G.H., Ann.Rev.Biophysics and Bioengineering, 5, 77-117, 1976.
- Watson, M.E.E., Nucleic Acids Research, 12, pp 5145-5164, 1984
- Winacker E.-L., From Genes to Clones, VCH Verlagsgesellschaft, Weinheim, Germany, 1987
- Zabeau, M. and Stanley, K.,EMBO J, 1, 1217-1224, 1982.
- Zaks, A., Empie, M. and Gross, A., Trends in Biotechnology 6, 272-275, 1988.
- Zell, R. and Fritz, H., EMBO J 6, 1809-1815, 1987.

CLAIMS

1. A cutinase derivative comprising a cutinase, preferably of natural origin, especially of fungal origin, particularly of Fusarium solani, quite particularly of F. solani pisi, which: 1) has, at its N-terminal end, a foreign amino acid sequence and/or 2) is not glycosylated.

2. The cutinase derivative of claim 1 which is not glycosylated and was secreted by a transformed prokaryotic cell, preferably E. coli.

3. The cutinase derivative of claim 1 which includes, at its N-terminal end, a foreign bridging sequence, preferably of about 10 to 35 amino acids in length, especially about 12 to 25 amino acids in length, particularly comprising a C-terminal portion of a propeptide segment of the cutinase of at least 5 amino acids, quite particularly comprising a part of the amino acid sequence of amino acids 17 to 32 of the cutinase of Fig. 1.

4. The cutinase derivative of anyone of claims 1 to 3 having the amino acid sequence of amino acids 32 to 230 of Fig. 1.

5. A recombinant gene for transforming a cell, preferably a prokaryotic cell, particularly E. coli, to produce a pure cutinase, characterized by the following operably linked DNA fragments in the same transcriptional unit:

a) a first DNA sequence that is foreign to the cell and encodes the cutinase, preferably of natural origin, especially of fungal origin, particularly of F. solani, quite particularly of F. solani pisi;

b) a promoter, such as the P_{tac}, P_{trp}, P_{lac}, P_R or lambda P_L promoter, upstream of the first DNA sequence and capable of directing the expression of the first DNA sequence in the cell; and

c) a transcriptional terminator, such as the transcription termination signal of phage fd, downstream of the first DNA sequence.

6. The recombinant gene of claim 5 also characterized by a second DNA sequence encoding a signal peptide which enables the cutinase to be secreted from the cell with the signal peptide being clipped off the cutinase; the second DNA sequence being upstream of, and in the same reading frame as, the first DNA sequence and being downstream of and under the control of the promoter.

7. The recombinant gene of claim 6 further characterized by a third DNA sequence encoding a bridging sequence between the signal peptide and the cutinase and preferably including all or part of a peptidase recognition sequence which enables all or part of the bridging sequence to be clipped off the cutinase by one or more peptidases, preferably the same, one or more peptidases which clip the signal peptide off the cutinase upon secretion of the cutinase; the third DNA sequence being upstream of, and in the same reading frame as, the first DNA sequence, downstream of the signal sequence and under the control of the promoter; the bridging sequence preferably encoding at least five amino acids of a C-terminal portion of a propeptide segment of the cutinase.

8. The recombinant gene of claim 7 wherein the first DNA sequence has the DNA sequence of Fig. 1 from nucleotide 362 to 961 and the second DNA sequence has the DNA sequence of Fig. 1 from nucleotide 268 to 317.

9. A cell, preferably a prokaryotic cell such as an E. coli cell, transformed with the recombinant gene of anyone of claims 5 to 8.

10. A pure cutinase, free of lipase.

11. A recombinant cutinase, preferably expressed by the transformed cell of claim 9.

12. The cutinase of anyone of claims 1 to 4, 10 or 11 which can hydrolyze an ester in the absence of an interface between the cutinase and the ester.

13. The cutinase of anyone of claims 1 to 4 or 10 to 12 which can hydrolyze an ester of a fatty acid of 16 to 18 carbon atoms.

14. An immobilized cutinase of anyone of claims 1 to 4 or 10 to 13.

15. A process of a) hydrolyzing or synthesizing esters, especially esters of fatty acids and/or fatty alcohols, or b) alcoholysis or acidolysis, or c) synthesizing a sugar ester, particularly for producing optically pure products, characterized by the use of the cutinase of anyone of claims 1 to 4 or 10 to 14 as an enzyme catalyst.

16. A method for producing a pure cutinase, preferably a cutinase derivative, characterized by growing a transformed cell of claim 9.

17. A method of dissolving a fat in an aqueous solution characterized by treating the fat with the cutinase of anyone of claims 1 to 4 or 10 to 14.

18. A detergent composition for dissolving a fat in an aqueous solution characterized by the cutinase of anyone of claims 1 to 4 or 10 to 14.

FIGURE 1

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        10         20         30         40         50
CGACGCGGGG TTCATACTTG ACCTTCCAAC ACCGTCGAAG CTCAGCCACT

        60         70         80         90        100
TCTCCAAGGC CCTCTGAGAA TCGGAGGATG GCTTGGTCGG TGGCGTCACC

       110        120        130        140        150
GTGGATGACA CGCTCGTGAA GAGGGAGTCT CATGGTGGCC GCGTCAAAGG

       160        170        180        190        200
AACCTGCGTT GCACAGTCCG GCAACGGCGC GGAGCTGGCT GAGAGAGTTG

       210        220        230        240        250
TTCTTGGGGC TGGTGTGCTG GTTGAGGACC ATGACGCCTC TTCCTTTTCA

       260        271        280        289
CTCTTTATCA TCTTCACC ATG AAA TTC TTC GCT CTC ACC ACA
                    MET Lys Phe Phe Ala Leu Thr Thr

    298        307        316        325
CTT CTC GCC GCC ACG GCT TCG GCT|CTG CCT ACT TCT AAC
Leu Leu Ala Ala Thr Ala Ser Ala|Leu Pro Thr Ser Asn

334        343        352        361↓       370
CCT GCT CAG GAG CTT GAG GCG CGC CAG CTT|GGT AGA ACA
Pro Ala Gln Glu Leu Glu Ala Arg Gln Leu|Gly Arg Thr

       379        388        397        406
ACT CGC GAC GAT CTG ATC AAC GGC AAT AGC GCT TCC TGC
Thr Arg Asp Asp Leu Ile Asn Gly Asn Ser Ala Ser Cys

    415        424        433        442
GCC GAT GTC ATC TTC ATT TAT GCC CGA GGT TCA ACA GAG
Ala Asp Val Ile Phe Ile Tyr Ala Arg Gly Ser Thr Glu
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FIGURE 1 (continued 1)

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451         460         469         478         487
ACG GGC AAC TTG GGA ACT CTC GGT CCT AGC ATT GCC TCC
Thr Gly Asn Leu Gly Thr Leu Gly Pro Ser Ile Ala Ser

        496         505         514         523
AAC CTT GAG TCC GCC TTC GGC AAG GAC GGT GTC TGG ATT
Asn Leu Glu Ser Ala Phe Gly Lys Asp Gly Val Trp Ile

    532         541         550         559
CAG GGC GTT GGC GGT GCC TAC CGA GCC ACT CTT GGA GAC
Gln Gly Val Gly Gly Ala Tyr Arg Ala Thr Leu Gly Asp

568         577         586         595         604
AAT GCT CTC CCT CGC GGA ACC TCT AGC GCC GCA ATC AGG
Asn Ala Leu Pro Arg Gly Thr Ser Ser Ala Ala Ile Arg

        613         622         631         640
GAG ATG CTT GGT CTC TTC CAG CAG GCC AAC ACC AAG TGC
Glu MET Leu Gly Leu Phe Gln Gln Ala Asn Thr Lys Cys

    649         658         667         676
CCT GAC GCG ACT TTG ATC GCC GGT GGC TAC AGC CAG GGT
Pro Asp Ala Thr Leu Ile Ala Gly Gly Tyr Ser Gln Gly

685         694         703         712         721
GCT GCA CTT GCA GCC GCC TCC ATC GAG GAC CTC GAC TCG
Ala Ala Leu Ala Ala Ala Ser Ile Glu Asp Leu Asp Ser

        730         739         748         757
GCC ATT CGT GAC AAG ATC GCC GGA ACT GTT CTG TTC GGC
Ala Ile Arg Asp Lys Ile Ala Gly Thr Val Leu Phe Gly
```

FIGURE 1 (continued 2)

```
    766         775         784         793
TAC ACC AAG AAC CTA CAG AAC CGT GGC CGA ATC CCC AAC
Tyr Thr Lys Asn Leu Gln Asn Arg Gly Arg Ile Pro Asn

802         811         820         829         838
TAC CCT GCC GAC AGG ACC AAG GTC TTC TGC AAT ACA GGG
Tyr Pro Ala Asp Arg Thr Lys Val Phe Cys Asn Thr Gly

        847         856         865         874
GAT CTC GTT TGT ACT GGT AGC TTG ATC GTT GCT GCA CCT
Asp Leu Val Cys Thr Gly Ser Leu Ile Val Ala Ala Pro

    883         892         901         910
CAC TTG GCT TAT GGT CCT GAT GCT CGT GGC CCT GCC CCT
His Leu Ala Tyr Gly Pro Asp Ala Arg Gly Pro Ala Pro

919         928         937         946         955
GAG TTC CTC ATC GAG AAG GTT CGG GCT GTC CGT GGT TCT
Glu Phe Leu Ile Glu Lys Val Arg Ala Val Arg Gly Ser

                971         981         991         1001
GCT TGA GGAGGATGAG AATTTTAGCA GGCGGGCCTG TTAATTATTG
Ala  .

     1011        1021        1031        1041        1051
CGAGGTTTCA AGTTTTTCTT TTGTGGAATA GCCATGATAG ATTGGTTCAA

     1061        1071        1081        1091        1101
CACTCAATCT ACTACAATGC CCATGTTTCA AATTAAAGAA GCAAAAAAAA

     1111        1120
AAAAAAAAAA CCGCGTCGAC
                 SalI
```

FIGURE 2

E1 : APTSNPALTTLLAATASALPTSNPAQELEARQL

E2 : none (i.e. direct S-C fusion)

E3 : APTSNPAQELEARQL

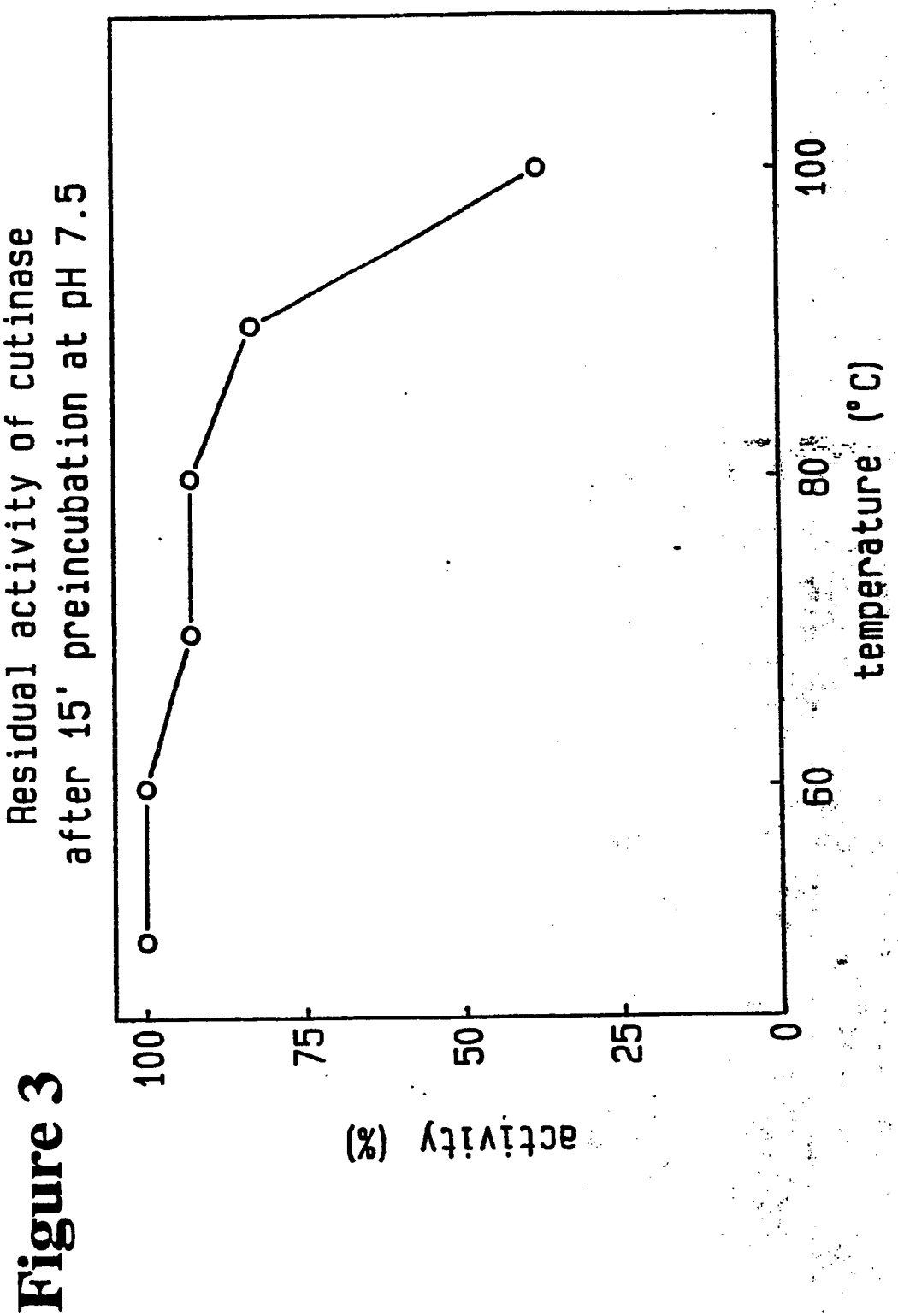

Residual activity of cutinase
after 15' preincubation at pH 7.5
activity (%)
100
75
50
25
0
60
80
100
temperature (°C)

Figure 3

Figure 4

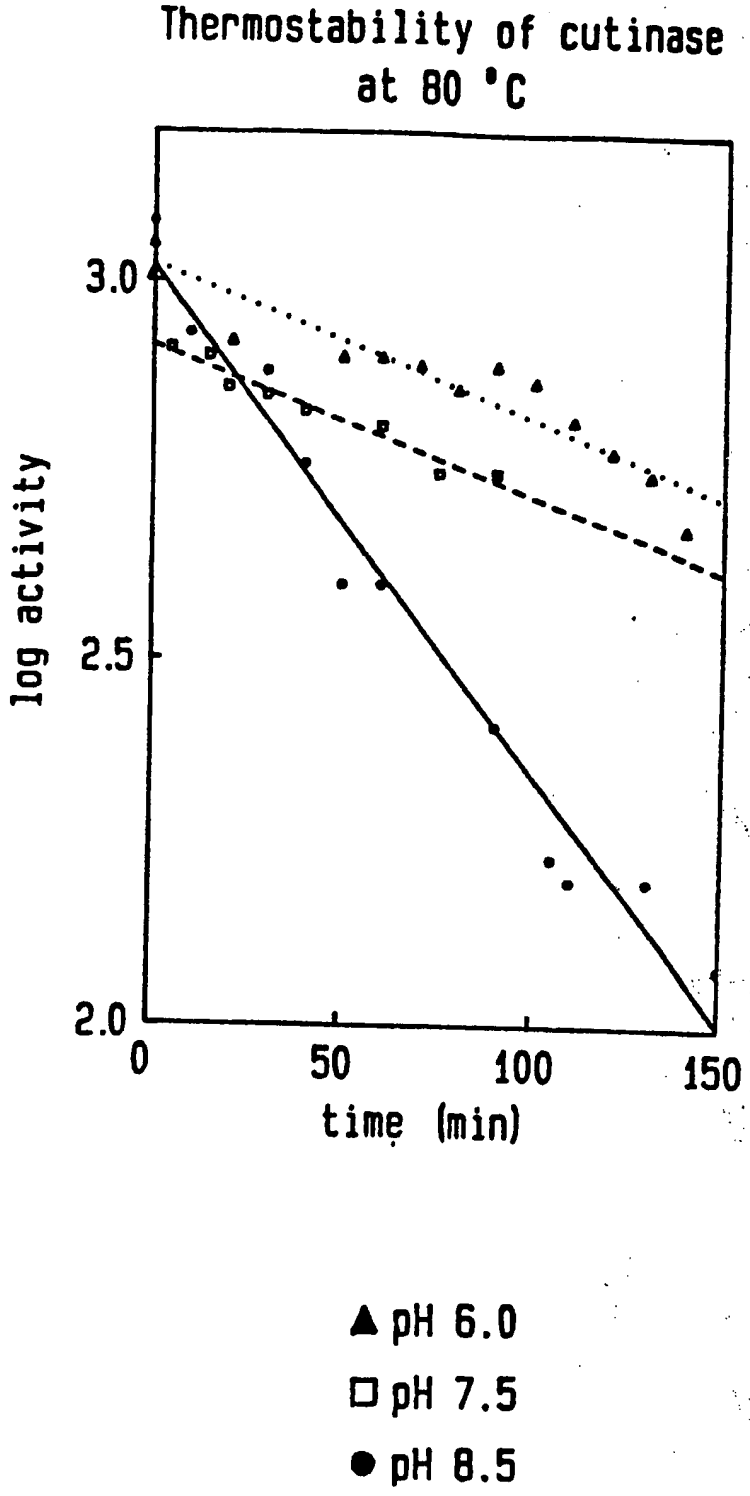
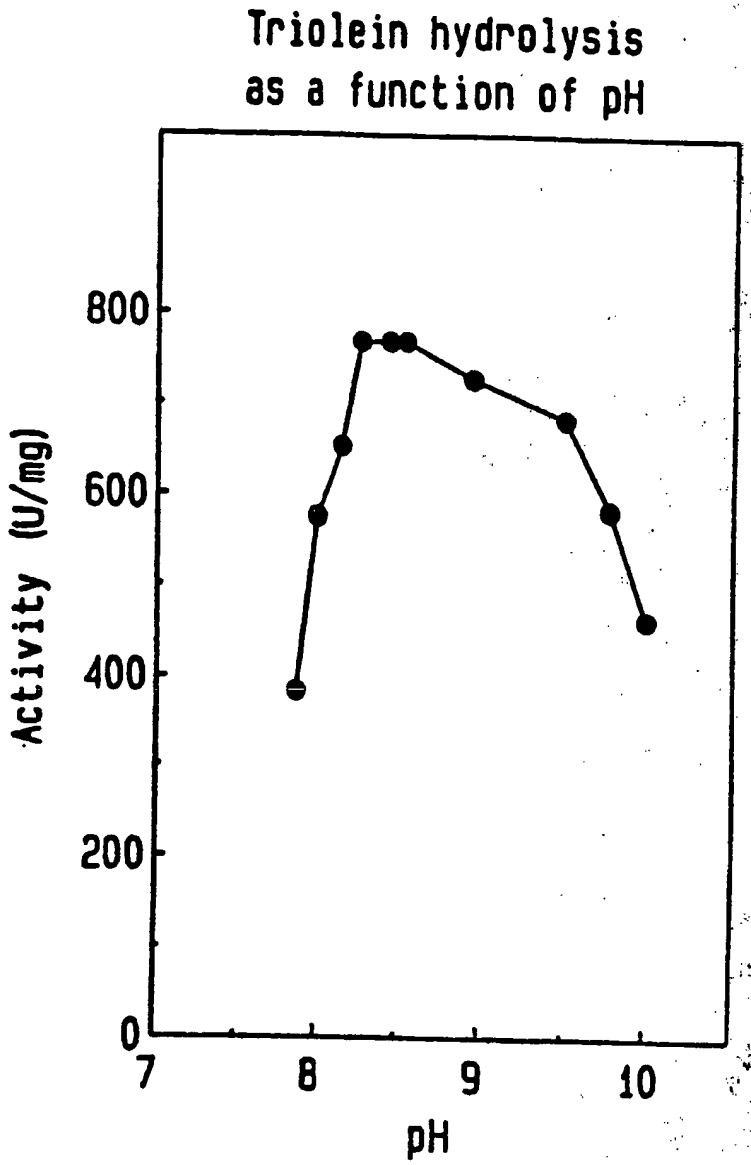

Thermostability of cutinase
at 80 °C
log activity
3.0
2.5
2.0
0
50
100
150
time (min)
▲ pH 6.0
□ pH 7.5
● pH 8.5

Figure 5


Triolein hydrolysis
as a function of pH
Activity (U/mg)
800
600
400
200
0
7
8
9
10
pH

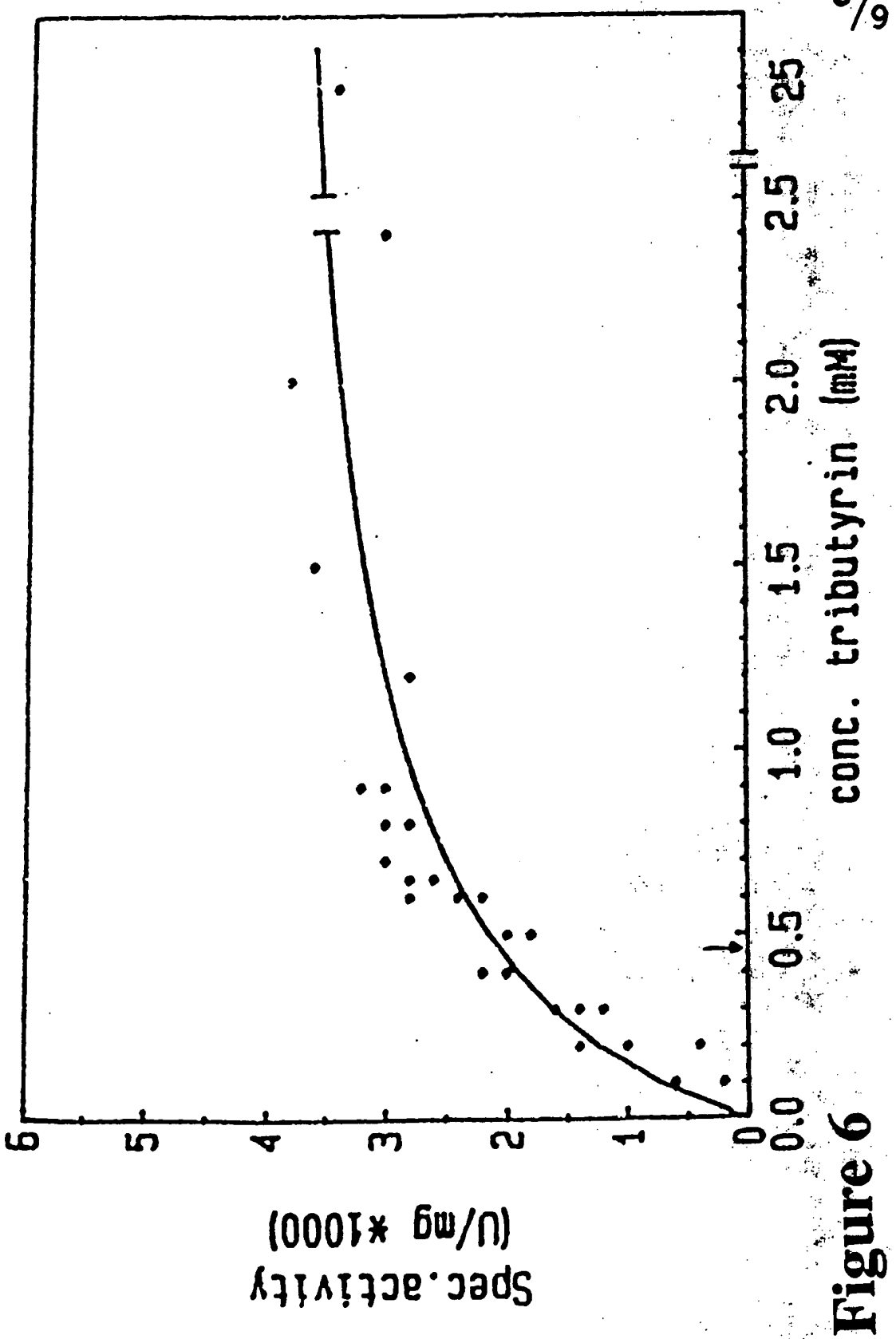
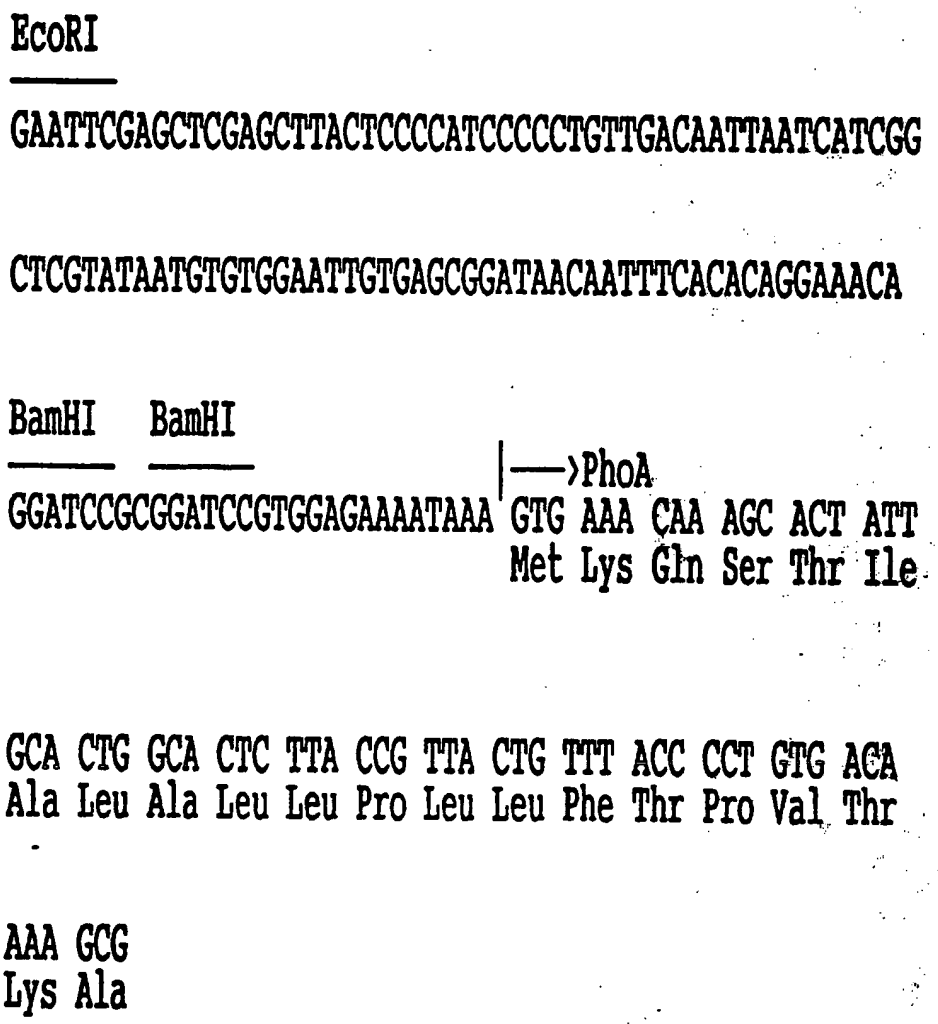

Spec.activity
(U/mg *1000)
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
25
conc. tributyrin (mM)

Figure 6

FIGURE 7


EcoRI
GAATTCGAGCTCGAGCTTACTCCCCATCCCCCTGTTGACAATTAATCATCGG
CTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACA
BamHI BamHI
PhoA
GGATCCGCGGATCCGTGGAGAAAATAAA GTG AAA CAA AGC ACT ATT
Met Lys Gln Ser Thr Ile
GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA
Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr
AAA GCG
Lys Ala

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/00289

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/55, C 12 N 9/18, C 12 P 7/62, C 11 D 3/386, // C 12 N 15/62

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC[5]	C 12 N, C 12 P, C 11 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Archives of Biochemistry and Biophysics, vol. 212, no. 1, November 1981, Academic Press, Inc., W.H. Flurkey et al.: "In vitro translation of cutinase mRNA: Evidence for a precursor form of an extracellular fungal enzyme", pages 154-161, see the whole article	1,2,10-13
Y	(cited in the application)	1,2,5,6,9, 10,11,16
	--	
X	Biochemistry, vol. 14, no. 13, 1975, R.E. Purdy et al.: "Hydrolysis of plant cuticle by plant pathogens. Purification, amino acid composition, and molecular weight of two isozymes of cutinase and a nonspecific	4,10,11-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd May 1990	02.07.90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	esterase from Fusarium solani f. pisi", pages 2824-2831, see the whole article	
	--	
Y	Proc. Natl. Acad. Sci. USA, vol. 81, July 1984, C.L. Soliday et al.: "Cloning and structure determination of cDNA for cutinase, and enzyme involved in fungal penetration of plants", pages 3939-3943, see the whole article (cited in the application)	1,2,5,6, 9-11,16
	--	
X	EP, A, 0268452 (GENENCOR INC.) 25 May 1988 see the whole document	10,12,13, 15-18
Y		1,2
	--	
X	WO, A, 88/09367 (GENENCOR, INC.) 1 December 1988 see the whole document (cited in the application)	10,18,5,6,9
	--	
A	Biochemistry, vol. 26, 1987, American Chemical Society, W.F. Ettinger et al.: "Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi", pages 7883-7892, see the whole article	
	--	
X	Archives of Biochemistry and Biophysics, vol. 263, no. 1, 15 May 1988, Academic Press, Inc., J. Sebastian et al.: "Purification of characterization of cutinase from a fluorescent Pseudomonas putida bacterial strain isolated from phyllosphere", ./.	10,13,15

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	pages 77-85, see the whole article --	
X	Chemical Abstracts, vol. 85, no. 19, 8 November 1976, (Columbus, Ohio, US), C.L. Soliday et al.: "Isolation and characterization of a cutinase from Fusarium roseum culmorum and its immunological comparison with cutinases from F. solani pisi", see page 191, abstract 139005d, & Arch, Biochem. Biophys. 1976, 176(1), 334-43	10,15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9000289 SA 34464

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/06/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0268452	25-05-88	AU-A- 8115387	26-05-88
		JP-A- 63283579	21-11-88
		AU-A- 7901387	09-06-88
WO-A- 8809367	01-12-88	EP-A- 0322429	05-07-89